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All three J-domain proteins of the *Escherichia coli* DnaK chaperone machinery are DNA binding proteins

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Abstract **DnaJ, DjlA and CbpA are the J-domain proteins of DnaK, the major Hsp70 of *Escherichia coli*. CbpA was originally discovered as a DNA binding protein. Here, we show that DNA binding is a property of DnaJ and DjlA as well. Of special interest in this respect is DjlA, as this cytoplasmic protein is membrane bound and, as shown here, its affinity for DNA is extremely high. The finding that all the three J-proteins of DnaK are DNA binding proteins sheds new light on the cellular activity of these proteins.**

Keywords: Heat shock response; DNA binding proteins; Chaperones

1. Introduction

Members of the Hsp70 family are chaperones that participate in protein folding, protection of proteins against unfolding, refolding of aggregated proteins, translocation of proteins across membranes and assist in protein degradation. They are present in all types of cells studied so far and in eukaryotes they are found in all the cellular compartments [1]. These chaperones perform their activities throughout allosteric changes achieved by repeated cycles of ATP hydrolysis. However, because their intrinsic ATPase activity is too slow, co-chaperones are required for enhanced ATPase cycles and for optimal chaperone activities [2–5]. One group of co-chaperones is comprised of members of the J-domain proteins. Several such proteins are present in every cell, all of which share a homologous domain (called J-domain) that plays an important role in their interaction with the Hsp70 counterpart and enhancement of its ATPase activity [6–8]. Apart from the J-domain, the rest of the protein sequence is not necessarily conserved among members of this large family.

In *Escherichia coli*, the most studied Hsp70 is the DnaK chaperone [1,9] required for protein folding and disaggregation especially at elevated temperatures [10–13]. Three *E. coli* J-proteins, DnaJ, DjlA and CbpA, have been shown to interact with DnaK and accelerate its ATPase activity [6,14–16]. DnaJ, encoded by a gene located in the same operon with *dnaK*, is the major J-protein of DnaK. It is a 41 kDa protein containing four distinct domains: an N-terminal J-domain, a glycine/phenylalanine domain whose function is not known, a zinc-finger domain shown to participate in enhancing the ATPase activity of DnaK and a C-terminal domain, which constitute about half of the protein and whose role is not clearly understood [17–20]. DjlA is a 30 kDa cytoplasmic protein that is anchored to the membrane by its N-terminal part. DjlA contain a C-terminal J-domain while the rest of the protein shares no homology with DnaJ. CbpA is a 34 kDa protein that contains an N-terminal J-domain and a C-terminal domain which are homologous to those of DnaJ. Its levels in the cytoplasm are markedly increased during late stationary phase and it is under the transcriptional regulation of σ^S, the stationary phase sigma factor [18,21].

CbpA was originally discovered by its ability to bind a synthetic curved DNA molecule (curved DNA binding protein A) and was shown later to be one of the major proteins constitute the bacterial nucleoid at stationary phase [21,22]. Here, we show that the ability to bind DNA is a property not only of CbpA, but also of DjlA and DnaJ. The binding is sequence non-specific with a K_d of 136, 48 and 455 nM, respectively, and is to any DNA molecule longer than 40 bases. This newly discovered activity of the J-domain proteins of the DnaK chaperone may advance our understanding of the role of these proteins in bacteria.

2. Materials and methods

2.1. Proteins

DnaJ, DjlA, CbpA, DnaK and HTS (Homoserine transsuccinylase) were purified as previously described [16,23]. GrpE was purchased from Stressgene (Cat. No. SPP-650).

DnaJΔJ-domain – The *dnaJ* gene, deleted for its first 71 codons, was cloned into the *Nde*I–*Bam*HI cut sites of the pET11a vector. The protein was purified like DnaJ with an additional step: Following gel filtration (as described [16,23]), the protein was dialyzed against 20 mM sodium-phosphate buffer, pH 6.8, 5 mM β-mercaptoethanol and loaded on a 20 ml HiPrep 16/10 SP-Sepharose FF (Amersham Pharmacia Biotech), which was equilibrated with the same buffer. The protein was eluted at a flow rate of 5 ml/min with a 200 ml of 0–1 M NaCl linear gradient. Rich fractions (as determined by SDS–PAGE) were collected and pooled. After concentration with YM-30 membrane (Amicon) glycerol was added to a final concentration of 10% and the protein solution was frozen at −70 °C.

The J-domain of DnaJ was purified with the Impact-CN system (New England Biolabs). The first 71 codons of *dnaJ* were cloned into the *Nde*I–*Sma*I cut sites of the pTYB2 vector and transformed into K12 strain ER2566 ($F^-\lambda^-$ *fhuA2* [*lon*] *ompT* lacZ::T7 gene*1 gal sulA11* α(*mcrC-mrr*)114::IS*10* R(*mcr-73*::miniTn*10*-TetS)2 R(*zgb-210*::Tn*10*) (TetS) *endA1* [*dcm*]). A fresh colony was used to inoculate 1.6 l of 2 × YT medium containing ampicillin (100 mg/l) at 30 °C and grow with aeration to about 3×10^8 cells ml^{-1} before induction with

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0.5 mM IPTG for 3 h. The cells were pelleted by centrifugation, frozen and resuspended in 30 ml of column buffer (20 mM Na-HEPES, pH 8.0, 500 mM NaCl, 1 mM EDTA) containing protease inhibitor cocktail (Sigma). All subsequent steps were performed at 4 °C. After the solution was stirred for 30 min, the cells were lysed by passage through a French Pressurizer cell. Following centrifugation at 23 000 × *g* for 30 min, the supernatant was loaded onto a 50 ml chitin column which was equilibrated with column buffer. The column was washed with 250 ml of column buffer followed by a quick wash (3 ml/min) with 150 ml of column buffer containing 50 mM DTT. The column was left at 20 °C for 24 h to allow on-column cleavage of the J-domain-Intein-CBD chimeric protein. Following elution with column buffer, the fractions which contained the J-domain (as determined by Tris–Tricine SDS–PAGE) were pooled and loaded on a YM-30 membrane (Amicon). The flow-through, which contained the J-domain was collected and concentrated with a YM-10 membrane. The protein solution (~500 μl) was diluted with 5 ml of 20 mM HEPES, pH 8.0, 1 mM EDTA and concentrated again using the same YM-10 membrane. Glycerol was added to a final concentration of 10% and the protein solution was frozen at −70 °C.

2.2. End labeling and Gel mobility shift assay

For DNA binding we used an end-labeled, 50 bp DNA molecule with the following sequence: GCCGAGGATCCAATTTGCTGT-CACTCCCATTTAAACGCTAGATGCTGTCA. End labeling was carried out in a reaction mixture of 40 μl containing 20 pmol ssDNA, 4 μl T4 × 10 PNK buffer (New England Biolabs), 6.25 mM DTT, 5 μl γ^{32}P-ATP and 1 μl T4 polynucleotide kinase (New England Biolabs). Following a 45 min incubation at 37 °C and inactivation of the kinase for 5 min at 90 °C, the complementary strand was added and the solution was slowly cooled to room temperature. The labeled DNA was extracted with phenol/chloroform (20 μl each) and the aqueous fraction was loaded over a G-25 microcentrifuge column (Amersham-Pharmacia). Typically, the DNA was labeled with ~1 000 000 cpm/μl.

For gel mobility shift assays, the radiolabeled DNA was diluted to 20 cpm/μl (260 cpm/μl for the λ-*Msp*I marker) in the reaction mixture (50 μl) and incubated with the protein for at least 2 h at 20 °C in a buffer containing 10 mM Tris–HCl, pH 7.6, 3 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mg/ml BSA and 0.02% NP-40. Glycerol was then added to a final concentration of 10% and 30 μl were loaded on a 7% polyacrylamide, 0.5 × TBE gels which were subjected to 300 V running for 30 min prior to sample loading. Running voltage was 250 V after the samples were loaded.

2.3. Co-immunoprecipitation

DnaJ and HTS (2 μl each) were incubated together for 2 h at 20 °C in 50 μl of PBS, 10 mM $MgCl_2$. Nine hundred and fifty microliters of PBS, 10 mM $MgCl_2$ and 3.5 μl of rabbit-anti-HTS serum were added for additional 30 min and then 35 μl of 1:1 ProteinA-sepharose:PBS were added. After 1.5 h of incubation the samples were centrifuged (5000 × *g*, 1 min) and washed three times with the same buffer. The precipitates were lyophilized (Speed-Vac) and 60 μl of protein sample buffer were added. Following 5 min boiling, the samples were centrifuged and equal amount of supernatants were loaded on 10% SDS–PAGE for Western blot analysis.

3. Results

3.1. DnaJ, DjlA and CbpA bind DNA non-specifically

It is known that CbpA is a DNA binding protein [22]. It was also shown to act as a J-protein for DnaK [16,18]. To determine whether DnaJ and DjlA, the two other J-proteins of DnaK, are DNA binding proteins as well, a gel mobility shift assays were performed. In this study we used a truncated derivative of DjlA. It is designated DjlΔTM, as it lacks the first 25 amino acids, which constitute the N-terminal periplasmic and trans-membrane domains. As a probe, we used a ^{32}P end labeled 50 bp synthetic double-stranded DNA molecule of random sequence. The results presented in Fig. 1 indicate that as expected, CbpA bound the DNA probe. Interestingly, DnaJ and DjlAΔTM also exhibited DNA binding activity. The negative controls, DnaK and GrpE, showed no considerable affinity for the DNA probe.

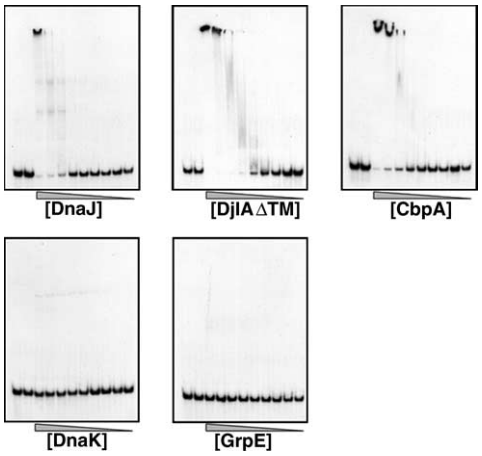

Fig. 1. DnaJ, DjlA and CbpA bind DNA. Gel mobility shift assays for DNA biding activity were carried out as described in Section 2 for DnaJ, DjlAΔTM, CbpA, DnaK and GrpE, as indicated. As a probe a 50 bp ^{32}P end labeled dsDNA was used. Proteins were added at 1:2 serial dilutions, starting from 2 μM. No protein was added in the first two left lanes of each gel.

To test if these proteins bind other sequences as well, we used a λ-*Msp*I DNA marker containing DNA fragments of different length and sequence (New England Biolabs). The DNA marker was ^{32}P-end labeled and gel mobility shift assays were carried out for binding by DnaJ, DjlAΔTM and CbpA and subjected to a gel mobility shift assay. As shown in

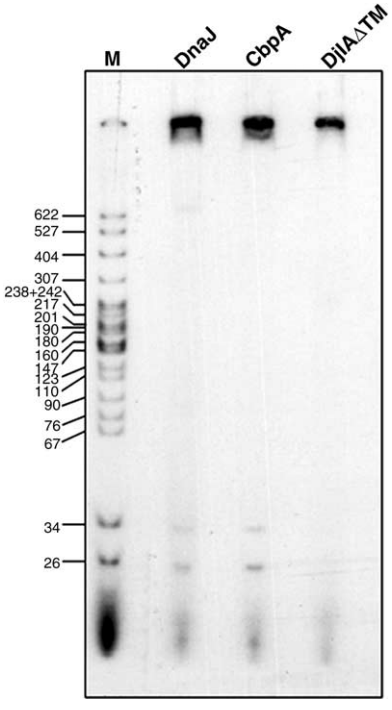

Fig. 2. DnaJ, DjlA and CbpA bind DNA fragments of various length and sequence. Gel mobility shift assay was carried out for DnaJ, DjlAΔTM and CbpA. As a probe a ^{32}P end labeled *Msp*I-λ marker was used. The first lane to the left contains only the marker (M), whereas the adjacent contains the marker with 2 μM of DnaJ, CbpA or DjlAΔTM, as designated.

Fig. 2, all three proteins bind DNA molecules of different length, regardless of their sequences. However, only DjlAΔTM bound the 34 and 26 bp fragments efficiently, while DnaJ and CbpA failed to do so. Additional experiments indicated that the lower limit for DnaJ and CbpA binding was 40 bp (data not shown).

As DnaJ, DjlA and CbpA share the conserved J-domain, we tested whether this domain is responsible for DNA binding in these proteins. To do that we constructed a DnaJ variant that lacks its J-domain (DnaJΔJ-domain) and purified the protein. In addition, we cloned and purified the N-terminal 71 amino acids of DnaJ, which constitute its J-domain. A comparison of DNA binding by the three proteins – complete DnaJ, DnaJΔJ-domain and J-domain – indicated that removal of the J-domain did not affect DNA binding. Compatible with this result was the finding that the J-domain by itself did not bind DNA significantly (Fig. 3). These results were unexpected, as the J-domain is common to all three J-proteins and, in addition, is positively charged (theoretical pI = 8.14). Yet, our result suggests that the J-domain is not responsible for the DNA binding activity of DnaJ, DjlA and CbpA.

3.2. DNA binding affinity of DnaJ, DjlA and CbpA

The affinity of the J-domain proteins to DNA was calculated from measurements of DNA binding at different protein concentrations. Gel mobility shift assays were performed as in Fig. 1, at various concentrations of each of the J-proteins and the unbound DNA was quantified using a phosphor imager. The results indicate (Fig. 4) that CbpA binds DNA with a K_d of 136 ± 4 nM, in good agreement with the previously published value (175 nM, [24]). Furthermore, the Hill coefficient equals 1.71 ± 0.09, indicating a considerable level of cooperative binding, as previously described. The cooperative binding of CbpA may result from its dimerization in solution [18]. DnaJ has the lowest affinity for DNA (K_d = 455 ± 40 nM) and the lowest n value. On the other hand, the affinity of DjlAΔTM for DNA was unusually high (K_d = 48 ± 4 nM). This finding ranks DjlA as one of the strongest sequence non-specific DNA binders among the known *E. coli* DNA binding proteins (HU – 25 nM, StpA – 118 nM, H-NS – 165 nM and Dps – 172 nM) [24].

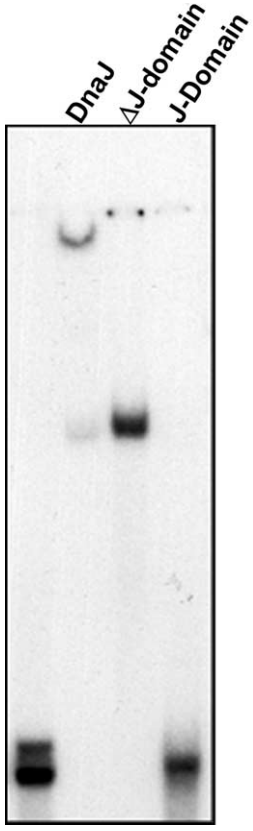

Fig. 3. *The J-domain is not responsible for DNA binding*. Gel mobility shift assay for DNA binding activity of DnaJ, DnaJΔJ-domain and the J-domain of DnaJ (2 μM each) was carried out as described in Section 2. A 50 bp ^{32}P end labeled dsDNA was used as a probe. The left lane contains only the probe.

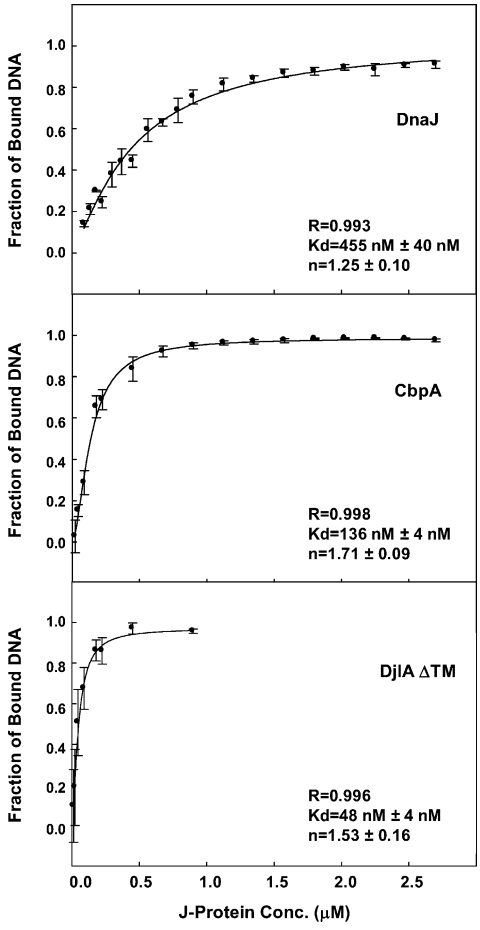

Fig. 4. Affinity of DnaJ, DjlA and CbpA for DNA. Gel mobility shift assays of DnaJ, DjlAΔTM and CbpA were carried out as in Fig. 1. The radioactivity of the unbound probe was quantified using phosphor imager (Pharmacia, Molecular Dynamics). Since in these experiments, the concentration of the probe was much lower than the assayed protein ($\sim 10^{-15}$ vs. $\sim 10^{-6}$ M, respectively), the unbound protein concentration practically equals the total protein concentration, allowing fitting the data to the Hill equation for cooperative binding: $\theta = (\theta_{max} \times [P]^n)/(K_d^n + [P]^n)$, where θ is the concentration of bound DNA θ_{max} is the maximal possible binding [P] is the concentration of the assayed protein and n is the Hill coefficient and a measure of cooperative binding. The indicated values are the means and standard deviations of three different measurements.

3.3. DNA binding and chaperone activity

The complex mechanism by which the DnaK chaperone machinery operates involves interaction between DnaK, GrpE, a substrate and one of the mentioned J-proteins. To test if DNA binding is affected by these protein interactions, we assayed for DNA binding by DnaJ, DjlA and CbpA in the presence of ATP and various combinations of DnaK, GrpE and substrate. We used Homoserine transsuccinylase (HTS) of *E. coli*, a thermolabile enzyme [23] which is a substrate of the DnaK machinery (unpublished data). The results (Fig. 5) indicate that the presence of DnaK or GrpE did not affect DNA binding by DnaJ. Conversely, the addition of DNA to the DnaK–GrpE–DnaJ complex did not affect the ATPase activity of DnaK (data not shown), suggesting that DNA binding to DnaJ does not affect the chaperone activity of the KJE system. Interestingly, the presence of HTS dramatically reduced DNA binding by DnaJ (Fig. 5) but not by CbpA and DjlA (not shown). The effect of HTS on DNA binding by DnaJ suggested that there is physical interaction between the two proteins. Indeed, we could show HTS binding to DnaJ by co-immunoprecipitation (Fig. 6). In this experiment, HTS and DnaJ were incubated together or alone before immunoprecipitation was performed with rabbit-anti-HTS serum. Western blot analysis was then performed with anti-DnaJ antibodies. The results indicated that following incubation with HTS, DnaJ could be pulled down with rabbit-anti-HTS antibodies and found in the precipitate (Fig. 6). The immunoblot indicated the presence of three bands, which reacted with the anti-DnaJ antibodies. All the three bands contained DnaJ, as determined by mass spectrometry. The finding that DnaJ binds to HTS is compatible with previous reports indicating substrate binding by DnaJ [19].

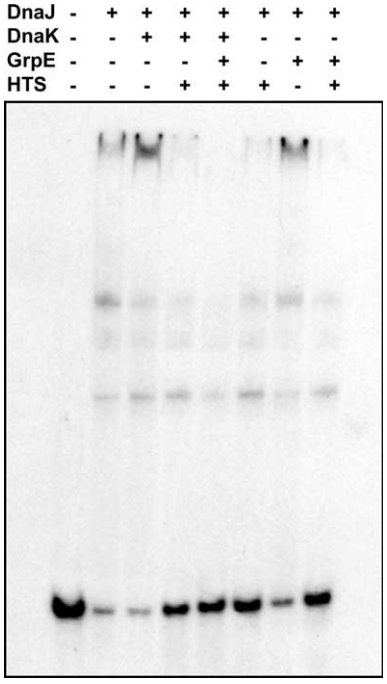

Fig. 5. HTS decreases the affinity of DnaJ for DNA. The 50 bp ^{32}P end labeled dsDNA probe was incubated in the reaction mixture (Section 2) with ATP (5 mM) and different combinations of DnaJ, DnaK, GrpE and HTS (2 μM each), as indicated. Gel mobility shift assay was performed as described in Fig. 1.

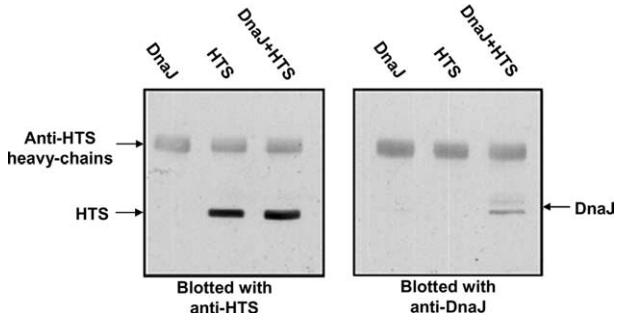

Fig. 6. Interaction of DnaJ with HTS. DnaJ was incubated with HTS and immunoprecipitation was carried out using rabbit-anti-HTS serum and ProteinA, as described in Section 2. The precipitates were loaded on a 10% SDS–PAGE and analyzed by Western blot using either rabbit-anti-HTS serum (left) or rabbit-anti-DnaJ antibodies (Stressgene) (right). The two gels also show the heavy-chains of the rabbit antibodies used for immunoprecipitation, which also react with the HRP-conjugated anti-rabbit antibodies used as secondary antibodies.

4. Discussion

DnaJ, DjlA and CbpA are the J-domain proteins of the DnaK chaperone complex in *E. coli*. CbpA was shown to bind DNA and was considered unique in being part of the *E. coli* nucleoid in stationary phase [21]. The results presented here indicate that all the J-proteins of DnaK bind DNA. Moreover, DNA binding affinity of DjlA is higher than this of CbpA (Fig. 4), and is among the highest among the known *E. coli* sequence non-specific DNA binding proteins.

The binding of DNA is not affected by the presence of the other members of the DnaK chaperone machinery (DnaK and GrpE, Fig. 5) and – conversely – DNA binding does not appear to affect the chaperone activity of this complex. Interestingly, the affinity of DnaJ to DNA was markedly reduced by the addition of a protein substrate. Thus, addition of HTS (a substrate of the DnaK chaperone), which interacts with DnaJ (Fig. 6) clearly decreased the binding of DNA. This property makes DnaJ unique among the J-proteins of the DnaK chaperone, and is compatible with previous reports that DnaJ binds to protein substrates.

The J-domain proteins function in the bacterial cell at different locations and at different growth stages. DnaJ is the major J-protein of DnaK and transcribed with it from the *dnaKJ* operon. It is a heat shock protein (under the control of σ32) and is considered to be responsible for most of the DnaK chaperone activities. In contrast, CbpA expression is σ^s dependent and its cytoplasmic concentration increases markedly during late stationary growth phase, reaching up to 3% of the total proteins [18,21,25]. Under these conditions CbpA becomes a major protein in the bacterial nucleoid, binding the bacterial chromosome at a ratio of about 1 molecule per 640 bp [21].

Here, we show that the affinity of DjlA for DNA is considerably higher than that of CbpA (K_d of 48 vs. 136 nM), as well as of many known *E. coli* sequence non-specific DNA binding proteins [24]. This finding is especially interesting in view of the fact that DjlA is anchored to the inner membrane and there-

fore, may serve as a linker between the bacterial chromosome and the membrane, or as a chaperone for proteins involved in DNA partitioning, known to take place on the membrane.

The affinity of DnaJ to DNA (K_d = 455 nM) is weaker than that of the two other J-proteins. Furthermore, we demonstrated that the DNA binding by DnaJ is reduced by its interaction with a protein substrate. Therefore, we assume that in the bacterial cell, which contains many substrates of the DnaK chaperone machinery, DnaJ would have little interaction with the bacterial chromosome.

The finding that all J-domain proteins of DnaK bind DNA in a sequence non-specific manner is unexpected and may shed new light on the physiological role of this important group of proteins and their function in bacterial protein quality control system.

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